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Transformation of Carbon Tetrachloride by Reduced Vitamin B_{12} in Aqueous Cysteine Solution

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Cysteine was studied as a reductant in carbon tetrachloride (CCl_4) transformation mediated by vitamin B_{12} at room temperature in the pH range of 4−14. The reaction proceeded in two consecutive phases: the initial phase was rapid but lasted only minutes before the slow subsequent phase started as B_{12} was inactivated, presumably due to nonreactive alkylcobalamin formation. The reduction of Co(III) to Co(II) was rate-limiting in the fast phase, whereas the decomposition of the alkylcobalamin may control the rate in the slow phase. B_{12r} was the reduced B_{12} species but exhibited little reactivity toward CCl_4 in the absence of cysteine; the reactive B_{12} species is hypothesized to be the pentacoordinated B_{12r}−cysteinate complex. Most of the CCl_4 was transformed to unidentified water-soluble products. The chloroform yield decreased with pH from 20% to nearly zero, whereas the carbon monoxide yield remained constant (3.2 ± 0.3%) with pH. These findings suggest that (1) the reductant controls both the kinetics and the mechanism of the reaction and should not be viewed simply as an electron donor, and (2) the B_{12} species involved in reductive biodehalogenation is likely to be either B_{12s} or B_{12r}−thiolate complexes.

Introduction

Chlorinated aliphatic hydrocarbons (CAHs) are of great environmental concern because they are widespread groundwater and soil contaminants, and many of them are known to be toxic or carcinogenic. Carbon tetrachloride (CCl_4), for example, is a potent liver toxin and a hepatocarcinogen (*1*). Of the principal transformation pathways for CAHs (*2, 3*), reductive dechlorination may be significant under anaerobic conditions. For many highly chlorinated hydrocarbons, reductive dechlorination is the only known biodegradation mechanism (*4*). CCl_4 and PCE, for instance, have not been found to degrade aerobically (*5*).

Although a few organisms are capable of deriving energy from reductive dechlorination of chlorocarbons (*6*−*10*), biological dechlorination reactions in most cases appear to be cometabolic; that is, the chlorinated pollutants are fortuitously transformed by enzymes of broad substrate specificity. The organisms do not benefit from the cometabolic processes. Ample evidence has indicated that the cell components responsible for cometabolic dechlorination reactions are most likely metallocoenzymes (*vide infra*), such as corrinoids (i.e., vitamin B_{12} derivatives).

Metallocoenzymes are presumed to be involved in cometabolic dechlorination for the following reasons. First, they are abundant in dechlorinating bacteria. Many anaerobic microorganisms, such as methanogens and acetogens, contain high levels of corrinoids (*11*−*13*); these organisms are often found to reductively dechlorinate CAHs (*14*−*17*). Second, metallocoenzymes such as corrinoids can abiotically catalyze reductive dechlorination reactions of CAHs (*18*−*21*) and aromatic chlorides (*20, 22*) in the presence of a bulk reductant. Third, these coenzymes are known to be heat-stable (*14*), which explains that the boiled cell extracts and enzymes of the dechlorinating organisms retain the dechlorination activities (*14, 17, 23*). Furthermore, coenzymatic and whole-cell dechlorination reactions usually exhibit similar product distribution patterns. For instance, of the three dichloroethylene (DCE) isomers, *cis*-1,2-DCE is the main dechlorination product of trichloroethylene (TCE) catalyzed by B_{12} (*20*), by a B_{12} holoenzyme purified from a methanogen (*23*), and by anaerobic microbial cultures in a contaminated field under methanogenic conditions [at the St. Joseph Superfund site, Michigan (*24*)]. Two metallocoenzymes, heme (*25*) and B_{12} (*23*), have been demonstrated to be the active components in the enzymes purified from the pure dechlorinating cultures.

In order to better understand cometabolic dechlorination reactions and to evaluate the fate of CAHs, it is necessary to understand the kinetics and the mechanisms of coenzyme-catalyzed dechlorination reactions. This prompted us to study coenzymatic dechlorination, using the transformation of CCl_4 in cysteine solution containing a catalytic amount of cobalamin as a model system. CCl_4 was chosen because it presents an environmental hazard and is highly prone to reductive transformation. Cysteine was selected as the representative thiol compound to study the effect of the reducing agent; it is a biologically abundant thiol, and its mild reducing strength [$E^\circ = -0.21$ V at pH 7 (*26*)] makes it a good contrast to titanium(III) citrate, a relatively strong reductant investigated in our previous study (*27*). Vitamin B_{12}, or cyanocobalamin, has been used as a model corrinoid in previous studies (*20, 27, 28*) and was therefore chosen to represent cobalamins for this study for comparison of results. Cobalamins are cobalt−corrin macrocycles containing an α-5,6-dimethylbenzimidazole ligand. The macrocycle is termed B_{12r} and B_{12s} when Co(III) in B_{12} is reduced to Co(II) and Co(I), respectively.

Although recent work (*27*) has shown that the two-step electron transfer (ET) model (*29, 30*) is too simplistic to adequately describe the complex interactions among the reductant, the cofactor, and the reaction intermediates, this model depicts the net flow of electrons and is conceptually useful as a starting point for developing a more detailed mechanistic model for the cysteine−B_{12}−

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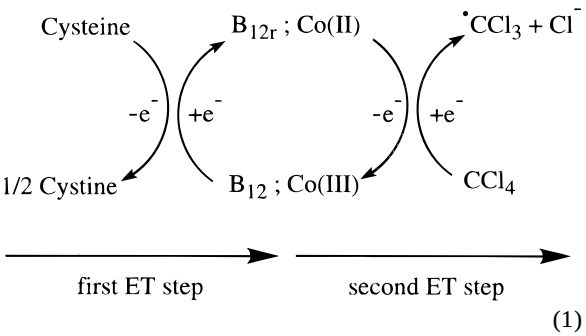
CCl_4 system (eq 1):



(1)

Specifically, we addressed the following questions in this study. (1) Which of the two electron transfer steps is rate-limiting? Although it has been suggested that the reaction of the substrate with the reduced cofactor can be rate-limiting (*28, 31*), in the case of a mild reducing agent like cysteine, the reduction of the cofactor may control the substrate degradation rate. (2) What is the reactive cobalamin species? The redox state of Co in thiol systems has been reported to be Co(II) (*17, 28, 32*); however, in their study of thiol autoxidation, Jacobsen et al. (*33, 34*) argued that Co(I) must be involved. (3) Does the activity of B_{12} change over time? Most kinetic studies to date focused on the *initial* pseudo-first-order rates (*20, 28*) while the inactivation of B_{12} during the reaction was not reported. (4) What is the role of cysteine? It was suggested that the role of the reducing agent is simply to reduce the coenzyme (*21*); i.e., the reducing agent is involved only in the first electron transfer step as shown in eq 1. However, this hypothesis is not supported by our results using either titanium(III) citrate (*27*) or cysteine as a reducing agent.

As a first step to resolve the reaction mechanism, we identified the rate-limiting step and hypothesized the reactive B_{12} species. We also identified a cause of the significant inactivation of B_{12} during CCl_4 transformation. The results are compared with those of the previous study in the titanium(III) solution, with focus on the role of the reducing agent on CCl_4 transformation.

Materials and Methods

Chemicals and Reagents. All chemicals were used as received without further purification: L-cysteine hydrochloride monohydrate (MicroSelect, >99%, Fluka, Ronkonkoma, NY), L-cysteine ethyl ester (>99%, Fluka), cyanocobalamin (vitamin B_{12}, CN-Cbl, 99%, Sigma, St. Louis, MO), cobinamide dicyanide (a corrinoid without the dimethylbenzimidazole ligand, Cbi, 95%, Sigma), aquocobalamin (B_{12a}, a B_{12} analogue with hydroxide rather than cyanide as the upper axial ligand, OH-Cbl, >96%, Fluka), carbon tetrachloride (>99%, Aldrich, Milwaukee, WI), and chloroform ($CHCl_3$, 99%, J. T. Baker, Phillipsburg, NJ). The calibration standards of CCl_4 and $CHCl_3$, the CCl_4 spike solution, and all O_2-free reagent stock solutions were prepared and stored as previously described (*27*). In most cases, the pH of the reaction mixture was maintained either by a separate pH buffer (0.2 M citrate, Tris-HCl, Borax, or carbonate/bicarbonate) or by cysteine itself [pK_a = 1.71, 8.33, and 10.78 (*35*)]; for pH 12 or greater, a 3.0 N NaOH solution was used to adjust the pH.

To test the reactivity of cob(II)alamin (B_{12r}) toward CCl_4 in the absence of a reducing agent, a cysteine-free B_{12r} solution was prepared based on the rapid and reversible reaction: $B_{12}(III) + B_{12s}(I) = 2\,B_{12r}(II)$ (equilibrium constant $\approx 10^{15}$; ref 36 and references cited therein). Titanium(III) citrate was added to a 360 μM B_{12} solution at pH 10.5 to completely reduce B_{12} to B_{12s} [i.e., reduce Co(III) to Co(I)]. A 12 N NaOH solution was then added dropwise to precipitate excess titanium(III) at pH above 11 (*27*). The precipitate was allowed to settle and was removed by transferring the supernatant through a 0.2-μm filter (Nalgene, Rochester, NY). A vitamin B_{12} solution of the same concentration but in slight stoichiometric excess was then added to the B_{12s} solution to give a stock solution of B_{12r} (final concentration = 320 μM) and B_{12}. The presence of a small amount of B_{12}, as confirmed by UV-visible spectroscopy, was required to ascertain that titanium(III) and B_{12s}(I), both reacting with CCl_4 (*27*), were absent in the solution and that B_{12r} was the only reduced Co species. In the absence of titanium(III) citrate and B_{12s}, CCl_4 transformation can be attributed to the only reduced species, B_{12r}.

Kinetic Study. All experiments were conducted at 22 ± 0.6 °C under anaerobic and light-excluded conditions. The pH range studied was 4−14. All experimental, sampling, and analytical procedures used were the same as described previously (*27*), except for the preparation of the bulk reducing agent solution. Each 2 oz. borosilicate reactor contained 60 mL of aqueous solution of L-cysteine (10 mM), B_{12} (nM range), and either NaOH or a 0.2 M pH buffer for pH control, leaving approximately 4 mL of 90% N_2/10% H_2 head space. Before and after each kinetic run, the pH of the reaction mixture was measured using an EA 940 expandable ion analyzer (Orion, Boston, MA); the two measurements normally agreed within 0.1 pH unit.

UV−Visible Spectra. Cob(III)alamin and cob(II)alamin (B_{12r}) were identified based on their characteristic absorption at 361 and 550 nm and at 312, 405, and 473 nm, respectively (*28, 35*). The formation of Co−C bonds in alkylcobinamides (but not alkylcobalamins) is indicated by an increase in absorption at approximately 460 nm (*36*); a peak at 464 nm has been determined for trichloromethylcobinamide, CCl_3−Cbi, which was formed in Cbi-catalyzed transformation of CCl_4 in titanium(III) citrate medium (*27*). The reduction of B_{12} to B_{12r} was carried out in 100 mM cysteine medium in the pH range of 8.3−12.5 using 8-mL amber screw-capped vials and monitored using an HP 8451A diode array spectrophotometer (Hewlett-Packard, Palo Alto, CA). The spectra were also measured upon introduction of 5 mM CCl_4 to the cysteine solution containing fully reduced B_{12r} and at pre-determined times during the reaction. A cysteine solution of identical concentration and pH was used as the reference for all measurements of B_{12} spectra.

Alteration of the cofactor and formation of alkylcobalt complexes during CCl_4 transformation were determined by comparing the UV-visible spectra of the Cbi(III) solution before adding cysteine and after completely reducing Cbi(III) to Cbi(II) with cysteine and spiking CCl_4 to trigger the reaction. The reaction was stopped in 20 min by adding a few drops of 30% H_2O_2. The measurements of the Cbi spectra were referenced to Milli-Q water.

Carbon Monoxide Yield. When CCl_4 was completely transformed, head space samples were withdrawn from the 8-mL amber vials and analyzed for carbon monoxide (CO) on an RGD2 reduction gas detector (Trace Analytical,

Menlo Park, CA). The CO calibration standards were purchased from Scott Specialty Gases (San Bernardino, CA).

$^{14}CCl_4$ Assays. A $^{14}CCl_4$ aqueous spike solution was prepared by adding nonradiolabeled CCl_4 to give a reaction solution containing approximately 200 nM CCl_4 and 1000 dpm/mL. The experimental conditions were as described in Kinetic Study. The reactions were carried out in 4-mL amber vials at pH 8.3 and 10.8. Upon complete CCl_4 transformation, three 1-mL aliquots were withdrawn from each vial with a 2-mL gas-tight syringe. Each aliquot was placed in a 20-mL clear scintillation counting bottle containing 0.2 mL of 1 M H_2SO_4, 0.1 mL of 1 N NaOH, or 0.1 mL of NaOH plus 15 mL of Universol (ICN, Irvine, CA) scintillation fluid, respectively. The acid and base bottles were then stripped with 99.9% N_2 for 20 min before adding 15 mL of scintillation cocktail. The residual radioactivity in each acid bottle (not removed by N_2 stripping) was taken as the water-soluble fraction. All samples were shaken and counted twice for 10 min using a Packard Tri-Carb 2500 TR scintillation analyzer (Meriden, CT).

Identification of Water-Soluble Products. An experiment was conducted to investigate whether intermediates of CCl_4 transformation were associated with cysteine and thus remained in the solution. Cysteine ethyl ester was used as the reducing agent instead of cysteine to facilitate extraction with methylene chloride (CH_2Cl_2). The reaction was conducted at pH 8.3 so that the CCl_4 degradation was fast relative to the hydrolysis of cysteine ethyl ester [the half-life ≈ 200 days at pH 8.3 and 1 h at pH 12, calculated using a reported hydrolysis rate constant (*37*)]. As all CCl_4 was transformed (within 2 h), the pH was adjusted to 12 and the aqueous solution was extracted with 1.5 mL of CH_2Cl_2. The extract was then concentrated to 0.2 mL, and the concentrate was analyzed for chlorinated products using a Finnigan (San Jose, CA) MAT TSQ 70 triple stage quadrupole mass spectrometer (chemical ionization with isobutane) and an HP 5890 GC/5970 mass selective detector (electron impact).

Results

Kinetic Data. Typical reaction curves, as shown in Figure 1, indicate a fast initial process followed by a slow subsequent process. The initial process fit to a zeroth-order (rate constant k_0', Figure 1a) model while the slow process fit to a first-order model (rate constant k_1', Figure 1b). The first phase was short (usually less than 20 min), and therefore only a few data points could be collected. Figure 1 also indicates that both k_0' and k_1' increased proportionally with B_{12} concentration, suggesting the reaction was first-order in B_{12} in both phases: the fast initial phase

$$-\frac{d[CCl_4]}{dt} = k_0' = k_1[B_{12}] \qquad (2)$$

and the slow subsequent phase

$$-\frac{d[CCl_4]}{dt} = k_1'[CCl_4] = k_2[CCl_4][B_{12}] \qquad (3)$$

k_1 and k_2 are the rate constants of the fast and the slow processes, respectively. When CCl_4 was respiked, the reaction continued to follow second-order kinetics at approximately the same rate (data not shown). In the absence of vitamin B_{12}, no appreciable CCl_4 transformation

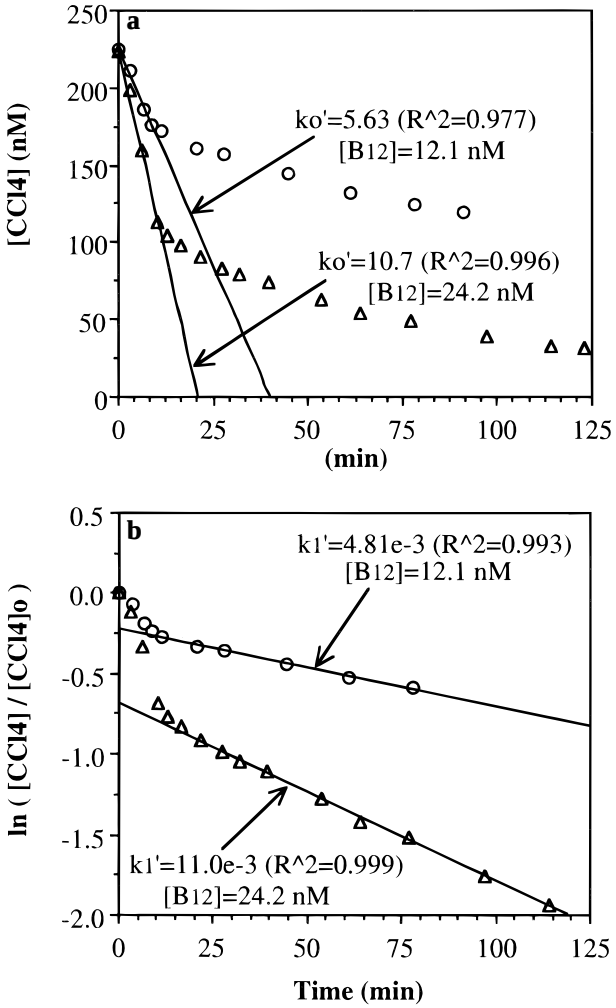


FIGURE 1. Typical CCl_4 reaction curves. The zeroth-order (rate constant k_0') and the first-order (rate constant k_1') kinetics before and after the transition are shown in panels a and b, respectively. [Cysteine] = 10.5 mM; pH = 10.7 (buffered by cysteine). Note that both rate constants are proportional to [B_{12}].

by cysteine was observed throughout the entire pH range tested (4−14).

The effect of pH on k_1 and k_2 is shown in Figure 2. CCl_4 was rapidly transformed only at pH greater than 8. Both k_1 and k_2 showed the same pH dependence: they first increased and then decreased with pH, with the maximum occurring at approximately pH 12 in both phases. Addition of a pH buffer, the concentration of which was about 20 times that of cysteine, did not affect k_1 and k_2.

The reactivity of B_{12r} toward CCl_4 in the presence and absence of cysteine was examined in two different solutions containing an identical number of electron equivalents: the first solution contained 320 μM B_{12r} as the only reduced species while the second contained 20 μM B_{12r} and 300 μM cysteine. In both systems, the reducing equivalent was in large excess of that of the CCl_4 added. The data in Figure 3 show that CCl_4 was not significantly degraded in the solution containing only B_{12r}; however, when both B_{12r} and cysteine were present, CCl_4 was completely transformed within 5 min. These results indicate that B_{12r} is rather nonreactive toward CCl_4 in the absence of cysteine and that the reactivity of B_{12r} is greatly promoted by cysteine.

The rate-limiting electron transfer step in eq 1 was identified by spectroscopic measurements. Figure 4 shows the spectral changes associated with the first electron

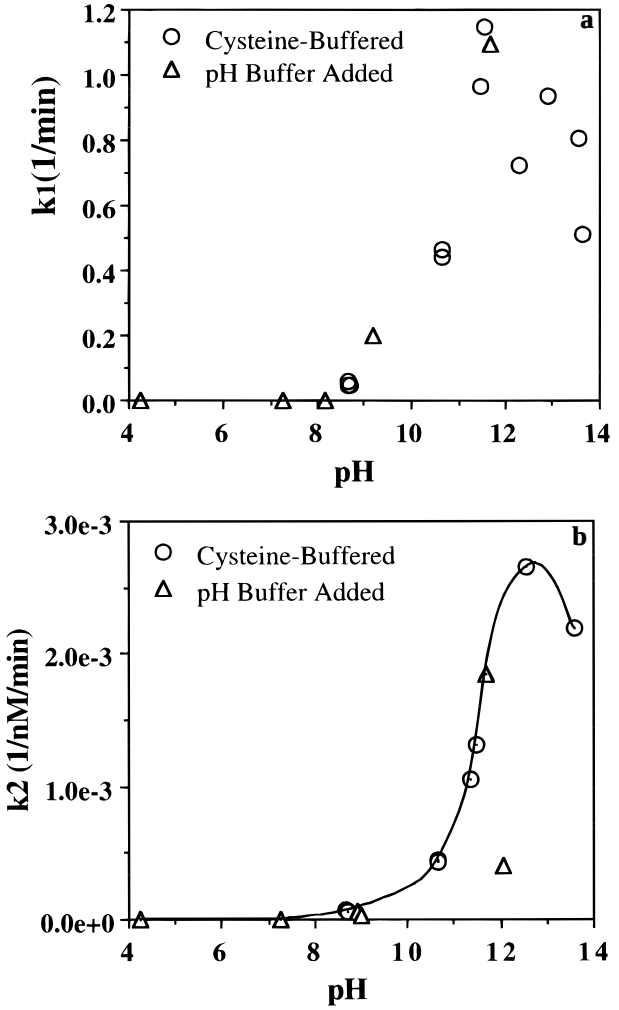


FIGURE 2. pH dependence of the rate constants. k_1 (a) and k_2 (b) were calculated using eqs 2 and 3, respectively. [Cysteine] = 10.5 mM; $[B_{12}]$ = 4–48 nM; $[CCl_4]_o$ = 200–560 nM.

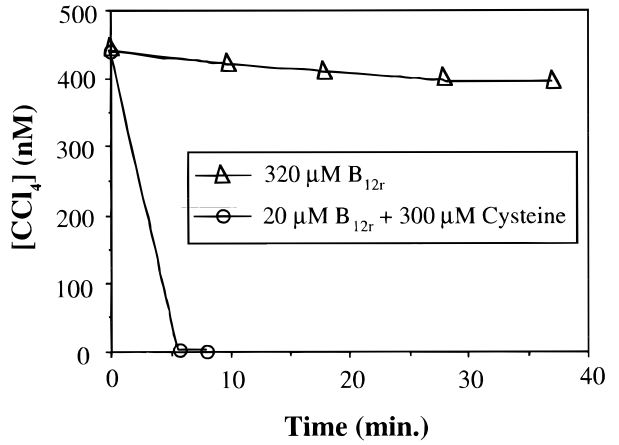


FIGURE 3. CCl_4 was rapidly transformed by vitamin B_{12r} only when cysteine was present. pH = 10.5 (0.2 M HCO_3^-/CO_3^{2-}); T = 22 °C. Note that the two systems had the same amount of electron equivalent.

transfer step. B_{12} was slowly reduced to B_{12r} in cysteine solution, as indicated by the gradual disappearance of the 362- and 550-nm absorption peaks and the concomitant appearance of the 312-, 408-, and 476-nm peaks. The time for complete reduction of Co(III) to Co(II), taken as the time required for the spectrum to stabilize, was pH-dependent. The reduction completed in 1 h at pH 9.5

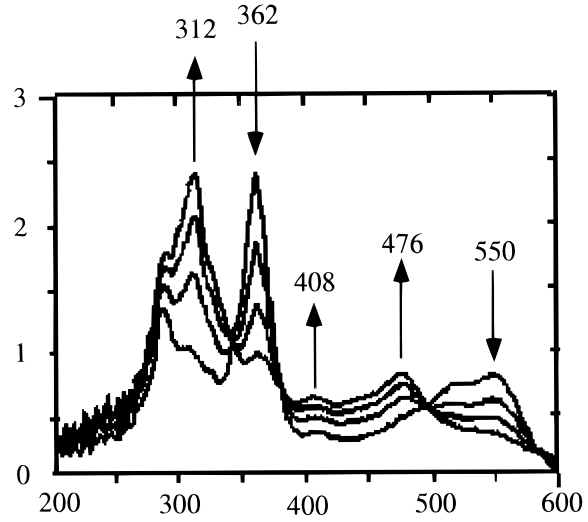


FIGURE 4. UV–visible spectroscopic changes of vitamin B_{12} (100 μM) during the reduction of Co(III) to Co(II) in 100 mM cysteine solution. pH = 9.5 (0.2 M borax buffer); T = 22 °C. The four spectra were taken at 1, 10, 30, and 60 min after B_{12} was added to the cysteine solution. As the two cob(III)alamin peaks (362 and 550 nm) gradually diminished, three cob(II)alamin peaks (312, 408, and 476 nm) increased correspondingly. The reduction was complete within 1 h, as indicated by the constant absorbances beyond 60 min. All measurements were referenced to a blank cysteine solution of identical concentration and pH.

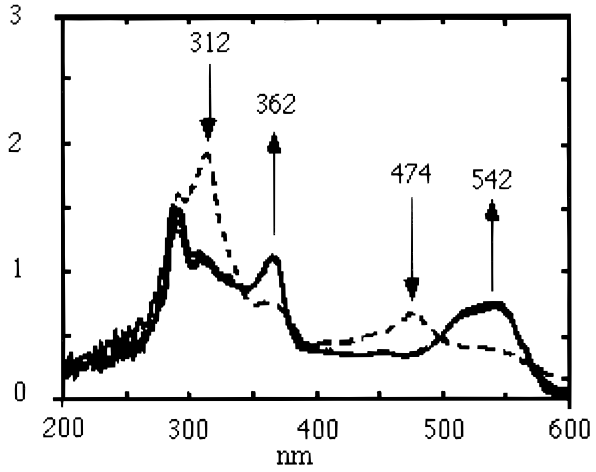


FIGURE 5. UV–visible spectral change of reduced cobalamin (90 μM) in 100 mM cysteine solution before (- - -) and 30 s after (—) the addition of CCl_4. Before CCl_4 was completely consumed, the spectrum did not change significantly, and Co remained as Co(III) throughout the reaction. $[CCl_4]_o$ = 5 mM; pH = 9.5; T = 22 °C. All measurements were referenced to a blank cysteine solution of identical concentration and pH.

(Figure 4) and 20 min at pH 12.5 (data not shown). Cob(I)alamin, which was detected in the titanium(III) medium (*27*), was not detected in the case of cysteine. The spectral changes in the second electron transfer step of eq 1 are shown in Figure 5. After B_{12} was fully reduced to B_{12r}, CCl_4 was added to the cysteine solution. The reaction vial was then shaken vigorously by hand for 30 s, and the spectra were measured immediately following shaking and periodically during the first hour of the reaction. In the first measurement taken (i.e., at t = 30 s after the addition of CCl_4), Co(II) was already completely oxidized to Co(III). In fact, the color change from yellow-orange back to pink occurred within 1 s of CCl_4 addition, indicating that the re-oxidation of the reduced B_{12} by CCl_4 was instantaneous.

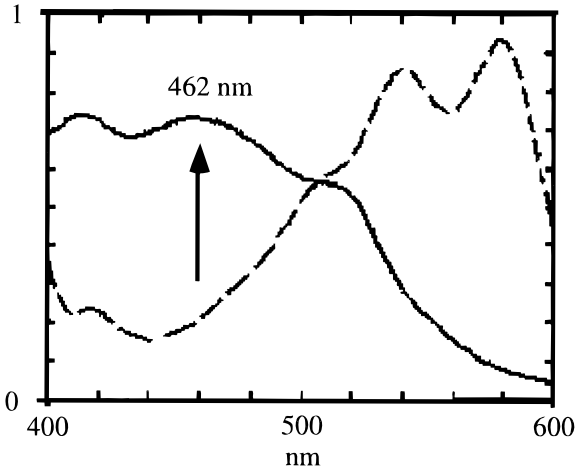


FIGURE 6. UV−visible spectra of cob(III)inamide (100 μM) before reduction by 10 mM cysteine (- - -) and after complete oxidation of cysteine (—) by CCl_4 (5 mM) and H_2O_2 (20 mM). pH = 10.7 (cysteine-buffered). The spectra were referenced to deoxygenated Milli-Q water.

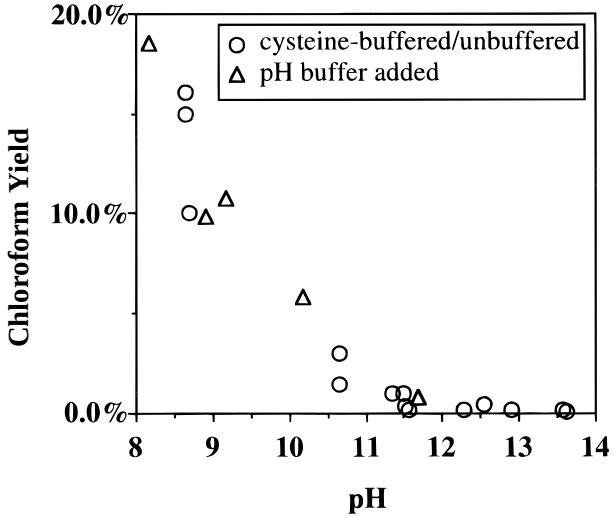


FIGURE 7. Chloroform yield decreased with increasing pH. All conditions were as described in the Kinetic Study section. Note that whether the system was buffered by an external pH buffer or by cysteine itself did not affect the $CHCl_3$ production.

Co remained predominantly as Co(III) in the first hour, and the spectrum did not change significantly except for the absorption peak at 290 nm, which increased gradually as the reaction proceeded.

Aquocobalamin (B_{12a}), the hydroxo (at pH > 7.8) congener of vitamin B_{12}, was tested in a separate experiment to compare the results to those obtained using B_{12}. B_{12a} was reduced more rapidly than vitamin B_{12} by cysteine: the color change to orange (B_{12r}) was noticeable within 10 s of cysteine addition, and the reduction was complete in about 5 min when followed spectrometrically. Introduction of CCl_4 immediately oxidized Co(II) back to Co(III), as in the case of vitamin B_{12}. This indicates that the reduction of both B_{12} and B_{12a} by cysteine was slower than the oxidation of the Co(II) species by CCl_4, and therefore Co(III) reduction should be rate-limiting in both cases.

The inactivation of B_{12} was investigated in an experiment using Cbi instead of Cbl as a catalyst for the detection of alkylcobalt intermediates (see Materials and Methods)—a hypothesized cause of B_{12} inactivation. Figure 6 compares the UV−visible spectra of Cbi(III) before reduction by cysteine and after reaction. The spectrum of Cbi(III) after reaction with CCl_4 followed by oxidation of cysteine was clearly different from the Cbi(III) spectrum before reduction. Since cysteine and cystine do not absorb strongly in the visible range, this spectral change is attributed to Cbi alteration during the reaction. And because the Co remained as Co(III), this alteration was most likely axial ligand substitution. Consistent with the hypothesis, a peak at 462 nm appeared in the spectrum taken after the addition of CCl_4 and cysteine oxidation, indicating that the Cbi was alkylated during CCl_4 transformation. Since alkylcobalt complexes are relatively stable, the CCl_4 transformation rate in the slow subsequent phase might be limited by the decomposition of the alkylcobalamin(s) formed during the reaction.

Product Identification. Two volatile products, $CHCl_3$ and CO, were quantified. $CHCl_3$ formation was determined during and at the end of the reaction. The $CHCl_3$ yield decreased with increasing pH from 20% at pH 8 to nearly zero at pH above 11 (Figure 7) and was not affected by the presence or absence of a pH buffer. CO was quantified after the CCl_4 was completely transformed at pH 8.3, 9.5, 10.7, and 12.5. The CO yield was 3.2 ± 0.3% and pH-independent. CO was produced only when CCl_4 was spiked and was absent in all controls.

TABLE 1

Product Distribution Obtained from $^{14}CCl_4$ Experiments[a]

pH	soluble (%)	volatile (%)	CO_2 (%)	CO (%)	$CHCl_3$ (%)
8.3	68	31	nd	3.2	18.2
10.8	93	6	nd	3.2	1.5

[a] $CHCl_3$ and CO yields accounted for most of the volatile fraction. nd, not detected above the background.

Experiments using $^{14}CCl_4$ were conducted at pH 8.3 and pH 10.8 (the two pK_a's of cysteine) to establish the carbon balance. The results along with the $CHCl_3$ and CO yields are summarized in Table 1. Water-soluble products account for most of the CCl_4 transformed. The 25% decrease in volatile products as pH increased from 8.3 to 10.8 can be largely accounted for by the decreased $CHCl_3$ yield at higher pH (Figure 7). The trace amounts of CO_2 detected were not significantly above the background levels.

Alkylation of B_{12} only explains a fraction of the soluble carbon mass: the detection of alkylcobalamin(s) (see Spectroscopic Data) indicates that a maximum of 20% (depending on the initial $[B_{12}]/[CCl_4]$ ratio) of the CCl_4 carbon was bonded to the Co(III). In an attempt to identify other water-soluble products, we searched for the thioethers CCl_3-SR and $CHCl_2$-SR. These thioethers may have formed from, for example, coupling of trichloromethyl radical, $^{\bullet}CCl_3$, and cysteine radical, $^{\bullet}$SR, similar to the S-alkylation reaction described earlier (*38, 39*):

$$^{\bullet}CCl_3 + {}^{\bullet}SR \rightarrow CCl_3\text{-}SR \qquad (4)$$

The disulfide dimer of cysteine ethyl ester was detected on the total ion chromatogram, which supports the formation of $^{\bullet}$SR. However, of the six unidentified peaks measured, none of them showed a characteristic chlorine pattern, and no molecular ions of the target thioethers were detected using chemical ionization with isobutane.

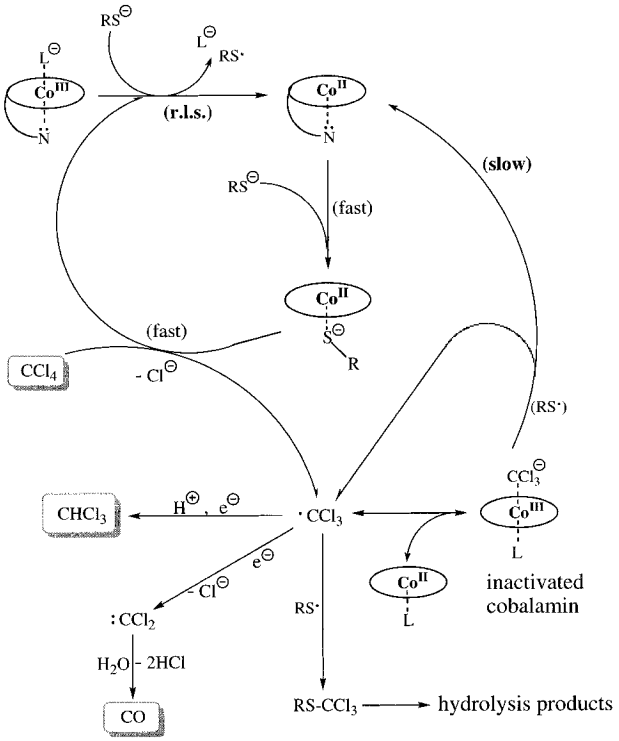


FIGURE 8. Hypothesized reaction scheme for CCl_4 transformation mediated by B_{12} in cysteine solution. Shadowed boxes are compounds measured in this study. r.l.s. = rate-limiting step; L = benzimidazole, RS^-, CN^-, OH^-, H_2O, or any other ligand.

Discussion

We have demonstrated that CCl_4 transformation by reduced B_{12} in cysteine solution proceeded in two distinct, successive phases. The reaction in the initial phase was rapid but lasted only minutes before the slow subsequent phase started as B_{12} was inactivated. A proposed reaction scheme is illustrated in Figure 8.

In the initial phase, the reduction of B_{12} to B_{12r} by cysteine controlled the overall degradation rate of CCl_4 (i.e., the first electron transfer step in eq 1 is the rate-limiting step). The redox conversion of the B_{12}/B_{12r} couple has been known to be relatively slow (*40*). The reduction of Co(III) to Co(II) completed within 1 h, whereas the reaction of the Co(II) species with CCl_4 (in excess) completed on the order of seconds. The observation that Co(III) remained the predominant species throughout the reaction before CCl_4 was depleted is also consistent with the postulate that the reduction of Co(III) by cysteine is rate-limiting in the initial reaction. Another piece of evidence supporting this postulate is that the B_{12} reduction rate and the initial CCl_4 transformation rate exhibited similar pH dependence: the reduction of B_{12} was approximately three times as fast at pH 12.5 as at pH 9.5 (completed in 20 vs 60 min), and so was the initial CCl_4 degradation rate (Figure 2a). The finding that reactions occurred rapidly only at pH above 8 indicates that the deprotonation of the sulfur atom ($pK_a = 8.3$) renders cysteine a much stronger reducing agent; that is, the cysteinate anion (RS^-) is presumably the species that reduces B_{12} to B_{12r}.

Since the second electron transfer step is fast relative to the first, B_{12r} would be expected to react with CCl_4 in seconds (Figure 5). However, the result in Figure 3 suggests otherwise. In the absence of cysteinate, B_{12r} showed little reactivity toward CCl_4; the rate at which CCl_4 was transformed was too slow to account for the reaction rate in the presence of both cysteinate and a catalytic amount of B_{12} at the same pH (Figure 1a). When cysteinate was present, however, all CCl_4 was degraded rapidly, indicating that cysteinate is also involved in the second electron transfer step. The finding that B_{12} requires cysteinate for high CCl_4 dechlorination activity suggests that it is not free B_{12r} but the B_{12r}–cysteinate complex that actually attacks CCl_4. Therefore, the rate-limiting step, the reduction of Co(III) to Co(II), must be followed by a fast coordination of cysteinate to form a pentacoordinated B_{12r}–cysteinate complex, B_{12r}–SR^-, which then rapidly reduces CCl_4 to generate $^{\bullet}CCl_3$ via a one-electron transfer. It is likely that the axial cysteinate ligand increases the electron density of the Co(II) and makes it a much stronger electron donor. In a separate experiment, we also observed a dependence of the B_{12r} dechlorination activity on thiol when cysteine was replaced with dithiothreitol (DTT) as the reducing agent: the disappearance of CCl_4 in a solution containing B_{12r} was observed only when DTT was present (unpublished data). This finding is supported by the rapid B_{12}-catalyzed CCl_4 transformation in DTT solution reported earlier (*28*). These observations suggest that other thiolate complexes of B_{12r} also have enhanced dechlorination activities. In addition, the reducing power or electron-donating tendency of the thiolate may affect the reactivity of the B_{12r}–thiolate complex. For example, PCE was transformed to TCE by vitamin B_{12} in a DTT medium within 1 week (*41*), but PCE dechlorination was negligible in a cysteine solution under identical conditions.

The UV-visible data confirm the earlier finding (*17, 28*) that thio reductants can generate only Co(II) but not Co(I). However, previous authors (*33, 34*) argued that Co(I) is the reacting species produced by thio reductants. These authors attributed the observed rapid dehalogenation in thiol solutions to Co(I) formation because B_{12s} is highly reactive toward organic halides (*42, 43*) whereas the reactivity of B_{12r}, calculated based on the rate laws reported by Blaser and Halpern (*44*), is too low to explain the fast dehalogenation. The discrepancy may be explained by hypothesizing that B_{12r}–thiolate complexes are far more reactive toward halocarbons than free B_{12r}; hence, the rate laws developed for B_{12r} in the absence of a reductant (*44*) may *not* be applied to systems containing a bulk reducing agent such as cysteinate. The observation that cobalamin was able to degrade 1,2-dichloroethane (DCA) with titanium(III) but not with cysteine (*17*) also suggests that nucleophilic attack by cob(I)alamin was probably not involved in a thio medium. This dechlorination reaction by B_{12} in cysteine solution presumably occurs via a one-electron transfer from the Co(II)–thiolate complex to the organic chloride. Alternatively, the enhanced reactivity of B_{12r} may be explained by assuming that a small fraction of the cobalamin exists as B_{12s} in a thiol medium. Pratt (*36*) suggested that thiolate–B_{12r} complexes may exhibit some characteristics of B_{12s}, similar to alkylcorrinoids where the electron density of Co(III) resembles that of Co(II) due to the strong σ-basicity of the axial carbanionic ligand. However, without direct evidence supporting the formation of B_{12s} in a thiol medium, our hypothesis remains that the B_{12r}–cysteinate complex is the reactive species that dechlorinates CCl_4 via a one-electron transfer.

The first-order dependence of the reaction rate on B_{12} concentration both before and after the kinetic transition (Figure 1) indicates that B_{12} was involved in CCl_4 trans-

formation in both the initial fast phase and the subsequent slow phase. The UV-visible spectra in Figure 6 further suggest that, during the initial phase, B_{12} was inactivated at least in part due to alkylation; therefore, the reaction in the slow phase might be limited by the decomposition of the alkylated cobalamin. One possible alkylation mechanism of B_{12} is the radical coupling reaction of B_{12r} and $\cdot CCl_3$, as for B_{12r} and methyl radical (*45*):

$$^{\bullet}CCl_3 + B_{12r} \rightarrow CCl_3\text{-Cbl} \quad (5)$$

CCl_3-Cbl is sufficiently stable to be prepared in the laboratory (*46*). The Co−C bonds in alkylcorrinoids are considered covalent and are more stable [bond enthalpy = 20−30 kcal/mol (*47*)] than the bonds between Co and Lewis bases such as hydroxide. The chemical inertness of alkylcobalt complexes has been noted. We previously showed that, in titanium(III) citrate medium, the slow cleavage of the Co−C bond limited the overall CCl_4 dechlorination rate (*27*). Jacobsen et al. (*33*, *34*) reported that the methyl and 5′-deoxyadenosyl cobalamins (Ado-Cbl, a.k.a. coenzyme B_{12}) and cobinamides catalyzed the autoxidation of thio reductants at rates 1−2 orders of magnitude lower than their aquo counterparts. For example, the rate constant for aquo-Cbl was approximately 50 times higher than that for methyl- and Ado-Cbl, a rate difference similar to that observed in the fast and slow phases in our experiments.

The breaking of the Co−C bond to regenerate of the reactive B_{12} species in the slow phase may occur via two different mechanisms: sterically induced thermal dissociation (*27*) and $^{\bullet}SR$ radical-assisted cleavage [eqs 6 and 7]. The latter is similar to the pathways proposed earlier (*39*, *48*):

$$CCl_3\text{-Cbl} + {}^{\bullet}SR \rightarrow CCl_3\text{-S-R} + B_{12r} \quad (6)$$

$$CCl_3\text{-Cbl} + {}^{\bullet}SR \rightarrow {}^{\bullet}CCl_3 + RS^- + B_{12} \quad (7)$$

Reductive cleavage of the Co−C bond by the reducing agent, which occurs in the titanium(III) citrate system (*27*), is unlikely in the case of cysteine because the redox potential of cysteine is too positive. Another possible cause of B_{12} inactivation is covalent modification of the macrocycle by reactive intermediates of CCl_4, as suggested elsewhere (*16*).

The water-soluble CCl_4 degradation products remain to be identified. Alkylation of B_{12} only accounts for less than 20% of the total CCl_4 carbon (provided that B_{12} was monoalkylated). Chlorinated thioethers, if formed, were either not extractable in detectable quantities using our procedures or unstable under the reaction conditions; for example, they might hydrolyze in a manner similar to other trichloromethyl sulfides in protic solvents (*49*). High yields of unidentified nonvolatile products of CCl_4 have been reported in similar systems (*16*, *18*, *28*). The low mass balance of CCl_4 was attributed to the highly nonspecific radical reactions (*16*), presumably involving $^{\bullet}CCl_3$. Complete mass balance of CCl_4 was obtained in the presence of an effective $^{\bullet}CCl_3$ scavenging agent; for example, when titanium(III) citrate was present at high pH to reduce $^{\bullet}CCl_3$ or when the medium was rich in organic material (*27*). Thiols are much milder reductants than titanium(III); therefore, radical reactions prevail, causing low yields of dechlorination products.

CO was confirmed to be a CCl_4 transformation product mediated by B_{12} in a thiol solution, as previously proposed (*28*). In titanium(III) citrate medium, 2−3 times higher CO yields were obtained (*19*). This suggests that the reducing agent may affect the formation of intermediates such as dichlorocarbene ($:CCl_2$), a presumed precursor for CO formation. It has been found (*27*) that titanium(III) citrate encourages the formation of trichloromethyl carbanion ($:CCl_3^-$), which may eliminate a Cl^- to give $:CCl_2$ and then hydrolyzed to yield CO. $CHCl_3$ was not likely to be an intermediate in CO formation because (i) base hydrolysis of $CHCl_3$ is negligible at pH below 10 under similar conditions (*27*) and (ii) CO was produced even at pH above 11 where very little $CHCl_3$ was formed.

In this study, an increasing transformation rate with pH (up to pH 12) in a thio reductant solution was observed and was reported for DTT [pH 5.0−8.5 (*28*)]; however, the reason why k_1 and k_2 decrease at pH beyond 12 remains unclear. Similar pH dependence of the reaction rate was also observed in the iron porphyrin-mediated oxidation of cysteine by CCl_4 (*41*) and molecular oxygen (*50*) and in corrinoid-catalyzed autoxidation of thiols (*33*), although the maxima appear at lower pH in those cases.

Compared with our previous study with titanium(III) citrate as a reducing agent (*27*), a much higher concentration of B_{12} is required to achieve a similar initial CCl_4 degradation rate with cysteine as the reducing agent, and the inactivation of B_{12} is more pronounced. Similar trends were also observed in the case of hematin (*41*).

Three conclusions may be drawn by comparing the results obtained using the two reducing agents, cysteine and titanium(III) citrate. First, the reducing agent controls the transformation kinetics and the pathways of these catalytic reactions and thus should not be viewed merely as an electron source to reduce the coenzyme, which was suggested elsewhere (*21*). In both reductant solutions, the reductant affects the reaction kinetics and the mechanism by generating different reactive species (e.g., B_{12s} vs B_{12r}−SR^-) and by reacting with the intermediates differently. We previously showed that titanium(III) can control both the CCl_4 degradation rate and the product distribution; this study further indicates that cysteinate is involved in both electron transfer steps and may react with intermediates such as $^{\bullet}CCl_3$ and CCl_3−Cbl via radical pathways. The reducing agent probably plays a more important role with polyhalomethanes such as CCl_4 as substrates than with vicinal polyhaloethanes. For polyhaloethanes, β-elimination is the favored pathway, and the ethylene production is nearly quantitative (*21*). For CCl_4, however, β-elimination is lacking and radical pathways predominate, and thus the mass balance is commonly low (*16*, *18*, *28*). An exception is in alkaline titanium(III) citrate solution where an effective radical-quenching mechanism involving the reducing agent exists and the $CHCl_3$ yield is nearly 100% (*27*).

Second, B_{12r} alone shows little dechlorination activity; therefore, if corrinoids are involved in reductive dechlorination reactions in a microorganism, it is likely that the organism either has strong reducing power to generate B_{12s} [$E°$ for $B_{12r}/B_{12s} = -0.53$ to -0.56 V vs SHE (*51*, *52*)] or has an active corrinoid enzyme with a thiolate, which could be a cysteine residue in the apoenzyme or a thio coenzyme, axially coordinating to the Co(II) atom of the B_{12}.

And third, corrinoids, although capable of catalyzing reductive dehalogenation reactions, may not be applicable to CAH destruction. Corrinoids were shown to be more

effective catalysts than most other metal tetrapyrroles (*53*) and have been proposed to treat industrial wastes containing halogenated compounds (*54*); however, the disposition of corrinoids to form stable Co−C bonds, an essential feature characteristic to all biological functions of B_{12} enzymes (*36*), may limit the effectiveness of corrinoids as catalysts in nonbiological treatment processes. During reductive dechlorination reactions, B_{12} is alkylated not only by saturated aliphatics such as CCl_4 but also by unsaturated chlorinated ethylenes (*20*). The cleavage of the Co−C bond limits the dechlorination kinetics in both reductant systems studied: in titanium(III) citrate solution, Co−C cleavage rate controls the overall CCl_4 transformation rate; in the cysteine system, formation of the Co−C bond results in the inactivation of the coenzyme.

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